



Hibernation induces changes in the metacerebral neurons of *Cornu aspersum*: distribution and co-localization of cytoskeletal and calcium-binding proteins

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Abstract

Pulmonate gastropods provide unique opportunities to examine physiological and biochemical adaptation strategies when cellular metabolic activity is reduced. In this study, cytochemical changes in metacerebral neurons of the cerebral ganglia were investigated in the garden snail *Cornu aspersum* during the hibernation phase. The immunocytochemical expression of three cytoskeletal markers: microtubule-associate protein 2-like (MAP-2-li), phosphorylated form of tau-like (P-Tau-li) and heavy subunit of neurofilaments-like (NF-H-li), and of two calcium-binding proteins: calmodulin-like (CaM-li) and parvalbumin-like (PV-li) was compared in active and hibernated snails. The immunopositivity for all the markers increased during hibernation versus activity in metacerebral neurons, with the notable exception of PV-li, which remained highly expressed during the whole annual cycle. Strongly positive aggregates of MAP-2-li and P-Tau-li were detected in the somata of hibernated snail neurons. P-Tau-li aggregates co-localized with CaM-li-labelled masses during hibernation. In addition, increased labelling of NF-H-li epitopes was associated with enhancement of CaM immunopositivity. These changes may reflect neural plasticity mechanisms mainly mediated by microtubule-associated proteins and CaM. Moreover, neuroprotective strategies may allow neurons to endure the prolonged hypometabolic conditions, taking into account that many of the functions controlled by the metacerebrum, such as feeding and movement, are suspended during hibernation. In this context, the molluscan ganglia model offers an easy opportunity to understand the molecular mechanisms behind these life cycle changes in cell physiology and to investigate possible cytological similarities among distantly related animals that adapt to the same environmental challenges through hibernation.

Keywords Hibernation · Gastropoda · Central nervous system · Immunohistochemistry · Neural plasticity

Abbreviations

bf	Barrier filter	MAP-2	Microtubule-associated protein 2
CaM	Calmodulin	MAP-2-li	Microtubule-associated protein 2-like
CaM-li	Calmodulin-like	NF	Neurofilament
CNS	Central nervous system	NF-H	Neurofilament-heavy
<i>C. Aspersum</i>	<i>Cornu aspersum</i>	NF-H-li	Neurofilament-heavy-like
DAB	Diaminobenzidine tetrahydrochloride	PHFs	Paired helical filaments
dm	Dichroic mirror	PV	Parvalbumin
excf	Excitation filter	PV-li	Parvalbumin-like
IFs	Intermediate filaments	PBS	Phosphate-buffered saline
MAPs	Microtubule-associated proteins	P-Tau	Phosphorylated-tau
		P-Tau-li	Phosphorylated-tau-like
		SE	Standard error

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Introduction

Living beings have evolved many diverse strategies to face changes in the environment and react to them. One of these mechanisms largely used to survive in adverse conditions is dormancy, in which metabolic activity is reduced and development, growth, movement and nutrition are temporarily suspended (Calixto 2015). Hibernation is considered as a deep dormancy associated with low temperatures that has been reported in several animals (Càceres 1997; Bickler and Buck 2007; Storey 2010) and has been repeatedly studied by our research group in vertebrates, particularly in mammals, using a morphological and histochemical approach (Bernocchi et al. 1986; Pisu et al. 1998; Cerri et al. 2009; Roda et al. 2017). The low metabolic rate associated with hibernation is particularly challenging for the nervous system, which requires a continuous supply of energy. Despite this, hibernating animals show an unmatched tolerance to hypometabolism thanks to numerous neuroprotective adaptations at the cell, tissue and system levels (Drew et al. 2001; Zhou et al. 2001; Stenzel-Poore et al. 2003; Dave et al. 2012).

Pulmonate gastropods belonging to the family Helicidae are considered model organisms to study histological, physiological and biochemical aspects of neural transmission, synaptic formation and plasticity and adaptation strategies during life cycle changes at tissue and cellular level (Korobtsov and Sakharov 1974; Antic et al. 2000; Balaban 2002; Azanza et al. 2008; Giachello et al. 2010; Matsuo and Ito 2011; Zatylny-Gaudin and Favrel 2014; Brenes et al. 2015). We have focused on *Cornu aspersum* (formerly known as *Helix aspersa*) for two main characteristics: (1) *C. aspersum* survives conditions of possible freezing through hibernation and (2) its nervous system anatomy is simple, characterized by a primitive organization of remarkably sized neurons concentrated in communicating ganglia, which are gathered in the cephalic portion of the body. Among these, the paired supraoesophageal cerebral ganglia receive sensory inputs and direct the activity of the other ganglia, from which axonal projections reach various organs in the visceral sac (Kerkut et al. 1975; Steffens 1980; Chase 2000). Each cerebral ganglion has been traditionally divided into three areas: the procererebrum, that receives the majority of olfactory information, the mesocerebrum, that controls reproductive behaviour, and the metacerebrum, that contains multiple neuronal populations associated with several functions such as feeding, movement and mating (Chase 2000). In the past, the metacerebrum has attracted particular interest due to the presence of two easily identifiable giant neurons: the giant cerebral neuron (GCN), a serotonergic cell that controls feeding behaviour, and the neuron C3, which is involved

in the tentacle withdrawal reflex (Pentreath et al. 1973; Hernádi et al. 1989; Chase and Tolloczko 1992; Prescott et al. 1997; Antic et al. 2000; Chase 2000).

The studies on *C. aspersum* can in turn contribute to the understanding of critical aspects of cell structure and function and of the nervous control of voluntary and autonomic behaviour (Balaban 2002). Hibernation, triggered by photoperiod, is characterized by a loss of water and food residues, thereby concentrating the haemolymph and removing ice nucleating agents, and by the production of low molecular weight and anti-freezing proteins (Ansart et al. 2001; Ansart and Vernon 2003). These processes allow the supercooling and the reduction of temperature below the freezing point without solidification. During hibernation, *C. aspersum* undergoes severe physiological and cytological modifications of neuronal cells of the cerebral ganglia, including the metacerebrum; changes in cytoskeletal proteins and neurotransmitters or signal molecules are reported (Vignola et al. 1995; Bernocchi et al. 1998; Pisu et al. 1999, 2000).

Similarly, differences in immunopositivity to numerous protein markers between activity and inactivity phases have been demonstrated in vertebrates. In particular, in several hibernating mammals cycles of torpor and arousal are present, in which body temperature oscillates between 37 °C and much lower values (Lust et al. 1989; Arendt and Bullmann 2013). The low metabolic rate during torpor is accompanied by decreased neuronal activity and altered connectivity through a reduction of the dendritic tree and consequently of synaptic contacts in different populations across the brain (Popov and Bocharova 1992; von der Ohe et al. 2006, 2007). This reversible remodelling and plasticity is in turn associated with molecular changes, particularly phosphorylation of enzymes and other proteins (Arendt 2004). Interestingly, this mechanism of reversible protein phosphorylation also affects the microtubule-associated protein tau, causing the aggregation of tau protein into paired helical filaments (PHFs) during torpor, as observed in Alzheimer's disease and other Tauopathies (Arendt et al. 2003; Härtig et al. 2007). PHF-like phosphorylation of tau in hibernating mammals, however, appears to be well tolerated and not associated with fibril formation; furthermore, it is fully reversible after arousal (Arendt et al. 2003). Therefore, it has been suggested that tau reversible hyper-phosphorylation might be a neuroprotective mechanism, conferring resistance to proteases, stabilizing cytoskeletal proteins so that they are readily used during arousal, and actively suppressing apoptosis (Su et al. 2008; Arendt and Bullmann 2013).

This research is aimed at deepening the knowledge about morphological and functional features of invertebrate neurons in the adaptation to natural conditions of hibernation (inactivity). In particular, using an antibody panel we evaluated cytochemical changes for cytoskeletal and calcium-binding proteins focusing on the large area of the cerebral

ganglion in which several functions are suspended in the hibernating *C. aspersum*, i.e. the metacerebrum.

The present study was designed with three objectives:

1. We first evaluated the immunoreactivity for microtubule-associated protein 2-like (MAP-2-li), phosphorylated MAP tau-like (P-Tau-li) and phosphorylated and non-phosphorylated heavy neurofilament-like (NF-H-li) proteins, based on their involvement in cellular stability and plasticity (Tucker 1990; Liu et al. 2004; Fletcher and Mullins 2010), and their intensive interaction that has been demonstrated both in vitro and in vivo (Leterrier et al. 1982).
2. We analysed the distribution and intensity of Calmodulin-like (CaM-li) protein since CaM has a conserved and fundamental role in signal transduction, synapse formation and protein modification (Kretsinger 1976; Klee and Vanaman 1982; Means et al. 1982; Hermann et al. 1991). We also compared the pattern with that of parvalbumin-like (PV-li) protein, another conserved calcium-binding protein that is involved in Ca^{2+} homeostasis and electric activity in neurons (Schwaller et al. 2002).
3. We searched for the colocalization of cytoskeletal markers and CaM-li, as their interaction is reported to regulate the dynamic balance between dimeric and polymerized forms of cytoskeletal components (Lee and Wolff 1984; Miyata et al. 1986). Since these activities are influenced by the degree of MAPs phosphorylation, which in turn is regulated by several kinases, including Ca^{2+} /CaM-dependent kinases (Schulman et al. 1985; Miyata et al. 1986; Sánchez et al. 2000; Gómez-Ramos et al. 2004; Iqbal et al. 2009), we tested the colocalization of CaM-li with P-Tau-li.

Materials and Methods

All experiments were conducted on sexually mature specimens (about 35 mm shell diameter) of the garden snail (*C. aspersum*) collected in their natural environment in Pavia, Northwest Italy. Ten active snails were collected in September (environmental temperature 23–27 °C) and maintained active (with food and mechanical stimulation by water drops) in the laboratory for 10 days before killing. Ten hibernated snails (with a complete epiphragm closing the shell) were collected in November (environmental temperature 4–9 °C) and kept in a cage in this condition until February, when they were killed. Ten snails collected as hibernated animals were fed and mechanically stimulated with water drops and awakened for additional 6 days.

The study was conducted under Italian Legislation of research protocol, in compliance with the European Council

Directive 2010/63/EU on the care and use of laboratory animals. All animals used in this research were treated according to institutional guidelines.

Fixation procedure, embedding and cutting

After dissection, cerebral ganglia were fixed in Carnoy's solution (absolute ethanol/chloroform/glacial acetic acid, 6:3:1) for 48 h with a change with fresh solution after 1 h, then placed in absolute ethanol, acetone, and finally embedded in paraplast (Paraplast X-tra, Sigma-Aldrich, St. Louis, MO, USA). Cerebral ganglia sections (8 µm thick) were obtained serially in the horizontal plane and collected on silan-coated slides; six sections per slide were obtained.

Paraplast-embedded sections of active and hibernated cerebral ganglia were deparaffinized in xylene, rehydrated in a decreasing ethanol series and rinsed in phosphate-buffered saline (PBS; Sigma).

Immunohistochemistry

To avoid possible staining procedure differences, all reactions were performed simultaneously on sections from active (including awakened specimens) and hibernated snails. For each selected marker, five slides (30 sections) from active snails, five from artificially awakened snails and five from hibernating snails were analysed. The figures show the most representative changes for each immunohistochemical reaction.

i. Fluorescence microscopy

- Single immunoreactions: sections were incubated overnight at 4 °C in dark moist chamber with commercial primary antibodies against CaM, MAP-2, P-Tau, NF-H (Table 1). After 12 h, sections were washed in PBS and incubated for 45 min with respective secondary antibodies Alexa Fluor 488-conjugated (1:100; Molecular Probes, Milan, Italy) and Alexa Fluor 594-conjugated (1:100; Molecular Probes, Milan, Italy), according to the type of reaction. After washing with PBS, nuclei were counterstained with 0.1 µg/mL Hoechst 33258 for 5 min and coverslips were lastly mounted in a drop of Mowiol (Calbiochem, San Diego, CA, USA).
- Double immunoreactions: As for CaM/P-Tau and CaM/NF-H, after overnight incubation at 4 °C with anti-CaM antibody, sections were washed twice in PBS for 5 min and then re-incubated overnight at 4 °C with a second primary antibody (Table 1). Thereafter, the protocol proceeded as for single immunoreactions.

Table 1 Primary antibodies and respective dilutions used for immunocytochemical experimental procedures

Antigen	Species	Manufacturer, catalog or lot No., RRID	Dilution
Microtubule-associated protein 2	Rabbit polyclonal IgG	Santa Cruz Biotechnology, H-300, Cat# sc-28197, RRID:AB_2269341	1:250
Phospho-tau	Rabbit monoclonal IgG	Cell Signalling Technology, D7U2P, Cat. #15013, RRID:AB_2728782	1:50
Calmodulin	Mouse monoclonal IgG	Abcam, Clone 2D1, Cat. # ab2860, RRID:AB_303362	1:20
	Rabbit antiserum	Swant, Cat. # 465, RRID:AB_10000349	1:1000
Parvalbumin	Mouse monoclonal IgG	Swant, Cat. # 235, RRID:AB_10000343	1:2500
Neurofilament 200 (phosphorylated and non-phosphorylated)	Mouse monoclonal IgG	Sigma, Clone N52, Cat. #N0142, RRID:AB_477257	1:100

Negative staining controls were incubated with PBS instead of the primary antibody, and no immunoreactivity was present in these sections.

An Olympus BX51 microscope equipped with a 100-W mercury lamp was used under the following conditions: 330–385-nm excitation filter (excf), 400-nm dichroic mirror (dm) and 420-nm barrier filter (bf) for Hoechst 33258; 450–480-nm excf, 500-nm dm and 515-nm bf for the fluorescence of Alexa 488; 540-nm excf, 580-nm dm and 620-nm bf for Alexa 594. Images were recorded with an Olympus MagniFire camera system and processed with the Olympus Cell F software.

ii. Light microscopy

Paraplast-embedded active and hibernating sections were deparaffinized in xylene, rehydrated in a decreasing ethanol series and rinsed in phosphate-buffered saline (PBS; Sigma). The endogenous peroxidases were suppressed by incubation of sections with 3% H₂O₂ in 10% methanol in PBS for 7 min. Sections were incubated for 20 min in normal serum at room temperature. Localization of CaM-li and PV-li was achieved by applying on cerebral ganglia sections a polyclonal rabbit anti-calmodulin and a monoclonal mouse anti-parvalbumin antibody, respectively (Table 1). Samples were then incubated overnight in a dark moist chamber. The following day, sections were sequentially incubated with biotinylated secondary antibodies (anti-rabbit, 1:200; Kit Vector Laboratories, Burlingame, CA, USA) for 30 min and horseradish peroxidase conjugated avidin–biotin complex (Kit Vector Laboratories) for 30 min at room temperature. Then, 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.01% H₂O₂ in Tris–HCl buffer (0.05 M, pH 7.6) was used as a chromogen. After each reaction step, sections were washed thoroughly in PBS, then dehydrated in ethanol, cleared in xylene and mounted in Eukitt (Kindler).

The slides were observed with an Olympus BX51 microscope, and the images were recorded with an Olympus Camedia C-5050 digital camera and stored on a PC. Corrections to brightness and contrast were made with Paint Shop Pro 7 (Jasc Software Inc).

For control staining, some sections were incubated with PBS instead of the primary antibody. No immunoreactivity was present in this condition.

Quantitative and statistical analyses

Immunofluorescence intensity was evaluated on acquired digitized images of active and hibernating sections under the same exposure time and avoiding any pixel saturation. Three slides (18 sections) coming from different cerebral ganglia were used for each phase (naturally active or artificially awakened snails versus hibernating snails) in the quantitative analysis for each marker considered (MAP-2-li, CaM-li, NF-H-li). The intensity of immunofluorescence staining, indicated as mean grey value, was measured using ImageJ particle analysis tool (ImageJ 1.51 s; NIH, Bethesda, MA, USA), which allows to identify particles of a defined size interval and measure individual feature profiles. The data were then recorded on Microsoft Office Excel spreadsheets and expressed as the total mean \pm standard error (SE). Statistical significance of the differences between hibernating and active animals was evaluated by unpaired Student's *t* test and indicated with a * for *p* value < 0.05.

For MAP-2-li, P-Tau-li and CaM-li proteins, the percentage of cells containing cytoplasmic aggregates was calculated considering three slides/phase/marker, counting the total number of metacerebral neurons and the number of cells with visible fluorescent masses in each section. Results were noted on Microsoft Office Excel spreadsheets and expressed as the mean % \pm SE. Unpaired Student's *t* test was then used to assess the statistical significance of the differences between activity and hibernation. Statistical significance is indicated with a * for *p* value < 0.05.

Results

A panel of antibodies (Table 1) was used here to label specific proteins, already reported in previous experimental studies characterizing mollusc nervous system, localized in *C. aspersum* cerebral ganglia. In particular, the

metacerebrum was examined, focusing on large neuron clusters (Fig. 1). Immunoreactivity-like patterns for all markers were simultaneously investigated in active and hibernated snail neurons. No differences were detected between specimens coming from naturally active or awakened snails; therefore, they were both considered as active specimens.

Immunofluorescence for microtubule-associated proteins

Immunofluorescence positivity for two MAP-like proteins was analysed in active and hibernated snail neurons: microtubule-associated protein 2-like (MAP-2-li) and phosphorylated tau-like (P-Tau-li) proteins (Fig. 2). A MAP-like protein with sequence similarity with human MAP-2 and Tau protein was discovered in the gastropod *Aplysia californica* (Shemesh et al. 2008); in the same species, antibodies raised for mammal antigens of the two MAPs labelled a 100- to 110-kDa protein, consistently with the tau-like proteins identified in *Drosophila melanogaster* and *Caenorhabditis elegans* (Shemesh et al. 2008).

MAP-2-li MAP-2 is one of the most abundant and best studied proteins of the MAP family; in the mammalian brain, it is expressed mainly in dendrites and axons (Matus 1988; Ludin and Matus 1993), where it is involved in the formation of cross-bindings between microtubules and neurofilaments and in microtubule assembly and stabilization (Leterrier et al. 1982; Weisshaar and Matus 1993; Sánchez et al. 2000).

Active snails showed MAP-2-li weakly stained neurons in the metacerebrum (Fig. 2a, b); high magnification images displayed a general diffuse immunoreactivity of cytoplasm or a higher intensity at one pole, likely at the level of the neurite cone (Fig. 2c). Hibernation caused an increase in labelling compared to active snails (Fig. 2d–f).

By evaluating all the immunostained sections of hibernating snails, a mean of 41% (SD 3.51) of metacerebral neurons was found to contain strongly positive aggregates scattered in the cytoplasm (Fig. 2e, f, see arrows). In the hibernated neuronal nuclei, Hoechst-stained heterochromatin masses were found compared with the fine granulated chromatin of active snails (compare Fig. 2f and 2c).

P-Tau-li Similarly to MAP-2, Tau possesses microtubule-binding domains and controls microtubule assembly and disassembly; in mammal neurons, it is primarily localized in axons (Dehmelt and Halpain 2004).

No specific positivity for P-Tau-li epitopes was observed in sections of active metacerebral neurons (Fig. 2g–i). Instead, a mean of 62% (SD 13.62) of hibernated neurons had intense positivity in the cytoplasm, which was concentrated in specific aggregates scattered throughout the cell cytoplasm (Fig. 2j–l, see arrows) and very similar to those observed for MAP-2-li.

Immunohistochemical reactions for calcium-binding proteins

CaM-li CaM is a calcium-binding protein that has been found in unicellular and multicellular eukaryote, in which it is primarily involved in cell signalling and Ca^{2+} -dependent protein phosphorylation. Homologs of vertebrate CaM proteins have been identified in all molluscs analysed to date, including several gastropods, and show a high degree of sequence conservation, with only 3 amino acid substitutions compared to vertebrates (Li et al. 2004, 2016; Simpson et al. 2005).

CaM-li immunoreactivity in *C. aspersum* showed a weak staining in most of the metacerebral neurons of active snails; some neurons had intense labelling (Fig. 3a). A marked

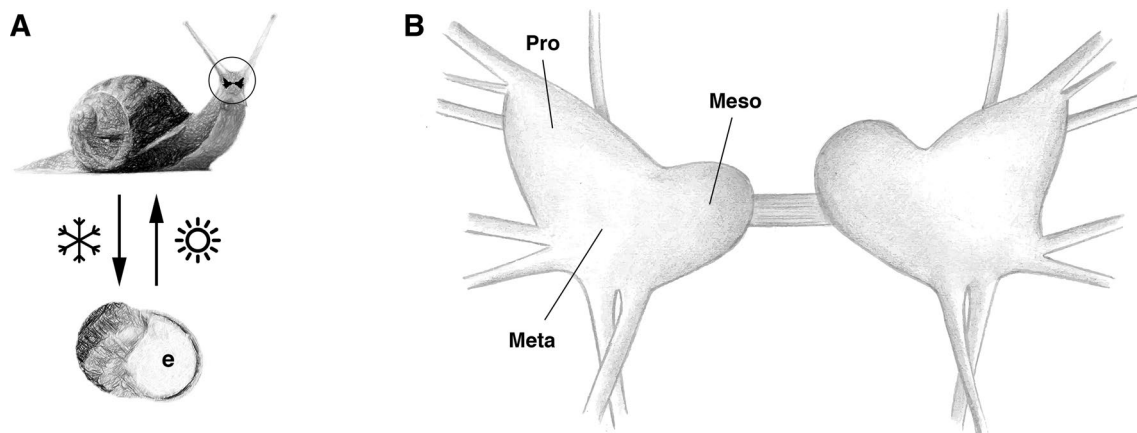


Fig. 1 **a** Simple representation of *C. aspersum* annual cycle: the garden snail produces a calcified membrane (epiphragm, e) and overwinters in protected niches called hibernacula. The position of the cerebral ganglia is indicated with a black circle. **b** Schematic drawing of

C. aspersum cerebral ganglia showing their three main portions: the procerebrum (Pro), the mesocerebrum (Meso) and the metacerebrum (Meta)

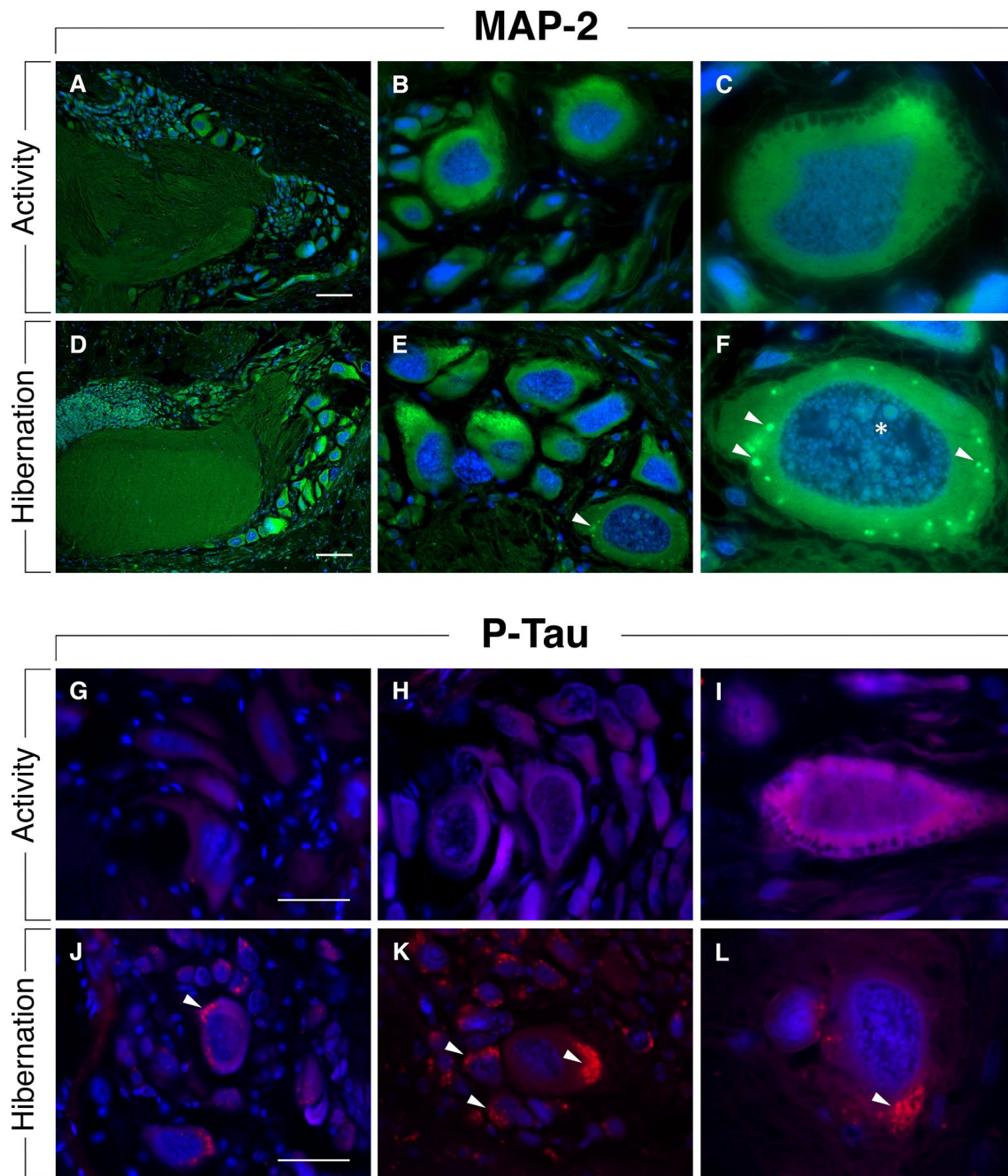


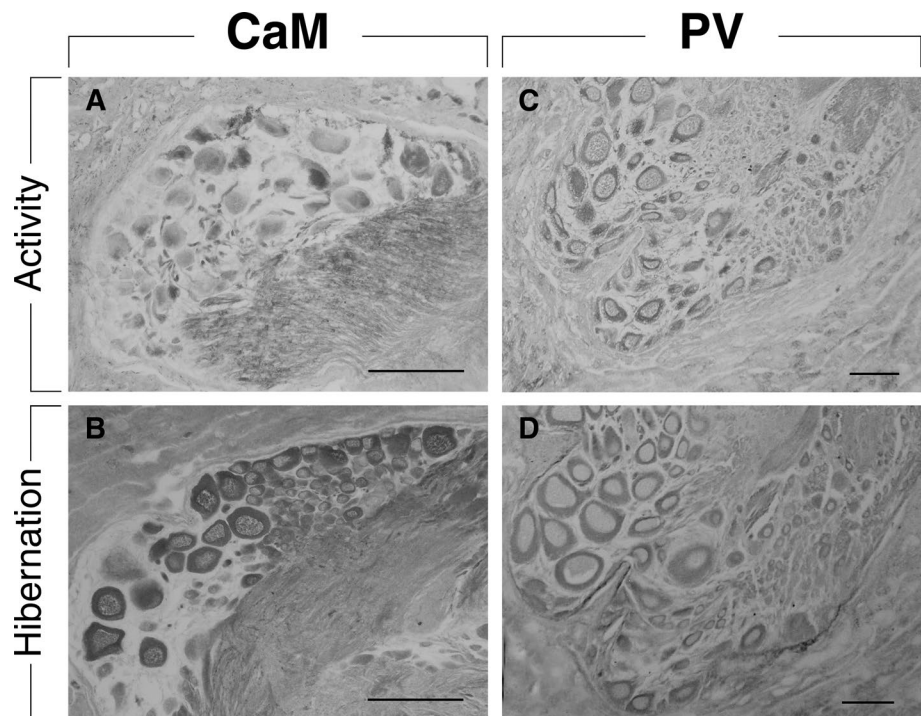
Fig. 2 Immunofluorescence staining for MAP-like proteins. **a–f** Immunoreactions to microtubule-associated protein 2-like (MAP-2-li) protein. Metacerebral active neurons showed a general, weak MAP-2-li immunopositivity (**a–c**) and were characterized by a euchromatic nucleus. Hibernated snails neurons displayed an enhanced immunopositivity (**d–f**), showing strongly MAP-2-li immunoreactive aggregates (**e, f**, arrowheads), scattered in the cytoplasm. The nuclei exhibited heterochromatin granulations (**f**, asterisk). **g–l** Metacerebral

neurons after phosphorylated tau-like (P-Tau-li) immunostaining. P-Tau-li immunoreactivity was pale in active neurons (**g–i**). On the other hand, hibernated neurons displayed intense P-Tau-li immunoreactivity (**j–l**); in particular, immunolabelled aggregates, clustered in cells cytoplasm, were clearly detectable (**j, k** and **l**, arrowheads). Nuclear granulations were also visible in several cells. *Objective magnifications:* 100× (**c, f, i, l**); 60× (**h, k**); 40× (**b, e, g, j**); 10× (**a, d**). MAP-2-li scale bar: 100 μm; P-Tau-li scale bar: 50 μm

increase in intensity and number of immunopositive large cells were observed in the hibernation phase (Fig. 3b). The findings agree with previous results obtained on the whole cerebral ganglion (Vignola et al. 1995).

PV-li PV is a low molecular weight protein involved in intracellular Ca^{2+} homeostasis. Previous studies have revealed the presence of a 40-kDa protein in neurons of *Helix pomatia* that possessed Ca^{2+} -binding properties and

Fig. 3 Immunostaining pattern for Ca^{2+} -binding proteins calmodulin-like (CaM-li) (a, b) and parvalbumin-like (PV-li) (c, d) in active and hibernated snails. A weak CaM-li immunolabelling was observed in some metacerebral neurons of active animals (a), while hibernating snails exhibited a diffuse increase in immunopositivity in the cytoplasm (b). All metacerebral neurons exhibited PV-li immunoreactivity in both active and inactive snails (c, d). *Objective magnifications:* 20 \times (a, b); 10 \times (c, d). Scale bar: 100 μm



was immunoreactive to antibodies against vertebrate parvalbumin (Kerschbaum et al. 1992, 1993).

All investigated cells of the metacerebrum were immunopositive to anti-parvalbumin antibodies during both activity and inactivity: no difference in PV-li immunoreactivity was observed between the two phases of the annual cycle (Fig. 3c, d).

Double immunofluorescence: CaM-li and P-Tau-li

During activity, CaM-li was present in the cytoplasm of neurons with weak labelling, although some neurons had labelling in a part of cytoplasm (Fig. 4b, e); no P-Tau-li immunoreactivity could be observed (Fig. 4a, d). In the hibernated snails, new epitopes were recognized by both antibodies (Fig. 4g–l): aggregates of P-Tau-li that colocalized with similar CaM-li aggregates were detected in the cytoplasm of 43% (mean value, SD 10.41) (Fig. 4j–l). As mentioned above, in some nuclei of hibernated neurons heterochromatin granulations were present (Fig. 4l).

Immunofluorescence for NF-H-li

Neurofilaments are structural components of nerve cell cytoskeleton involved in the maintenance of neuronal shape and axon calibre (Julien 1999). The phosphorylation of NF regulates neurofilament interaction and assembly, as well as interaction with other cytoskeletal elements and calcium-binding proteins (Sternberger and Sternberger 1983; Lee et al. 1987). Neuronal and non-neuronal intermediate

filaments were discovered in several molluscs (Weber et al. 1991; Way et al. 1992; Erber et al. 1998) including *C. aspersum* and *Helix pomatia*. Moreover, neurofilaments were observed and extracted from neurons of *Helisoma*, in which they positively reacted to anti-mammalian antibodies (Khan and Saleuddin 1987).

In sections from active ganglia, NF-H-li positivity was detected in the cytoplasm and nucleus of metacerebral neurons (Fig. 5a, b). The intensity of the immunoreaction markedly increased in the cytoplasm of hibernated snail neurons, including the giant C3 cell, with absence of positivity in the nuclei (Fig. 5c, d).

Double immunofluorescence: CaM-li and NF-H-li

The two markers colocalized in the neuronal cytoplasm of hibernated snail metacerebrum (Fig. 5e–g). Strongly labelled CaM-li immunostained aggregates were found mainly at cell periphery (likely axonal cone) of neurons (Fig. 5f). Moreover, there was an increase in NF-H-li immunopositivity in metacerebral neurons, particularly evident in the giant C3 neuron, in which heterochromatinic masses were also observed (Fig. 5d, e). No specific co-localization could be detected at the level of the CaM-li aggregates (Fig. 5g).

Statistical analysis

The quantitative evaluation of fluorescence intensity was performed on three markers (MAP-2-li, CaM-li and NF-H-li) for which we observed differences in the labelling

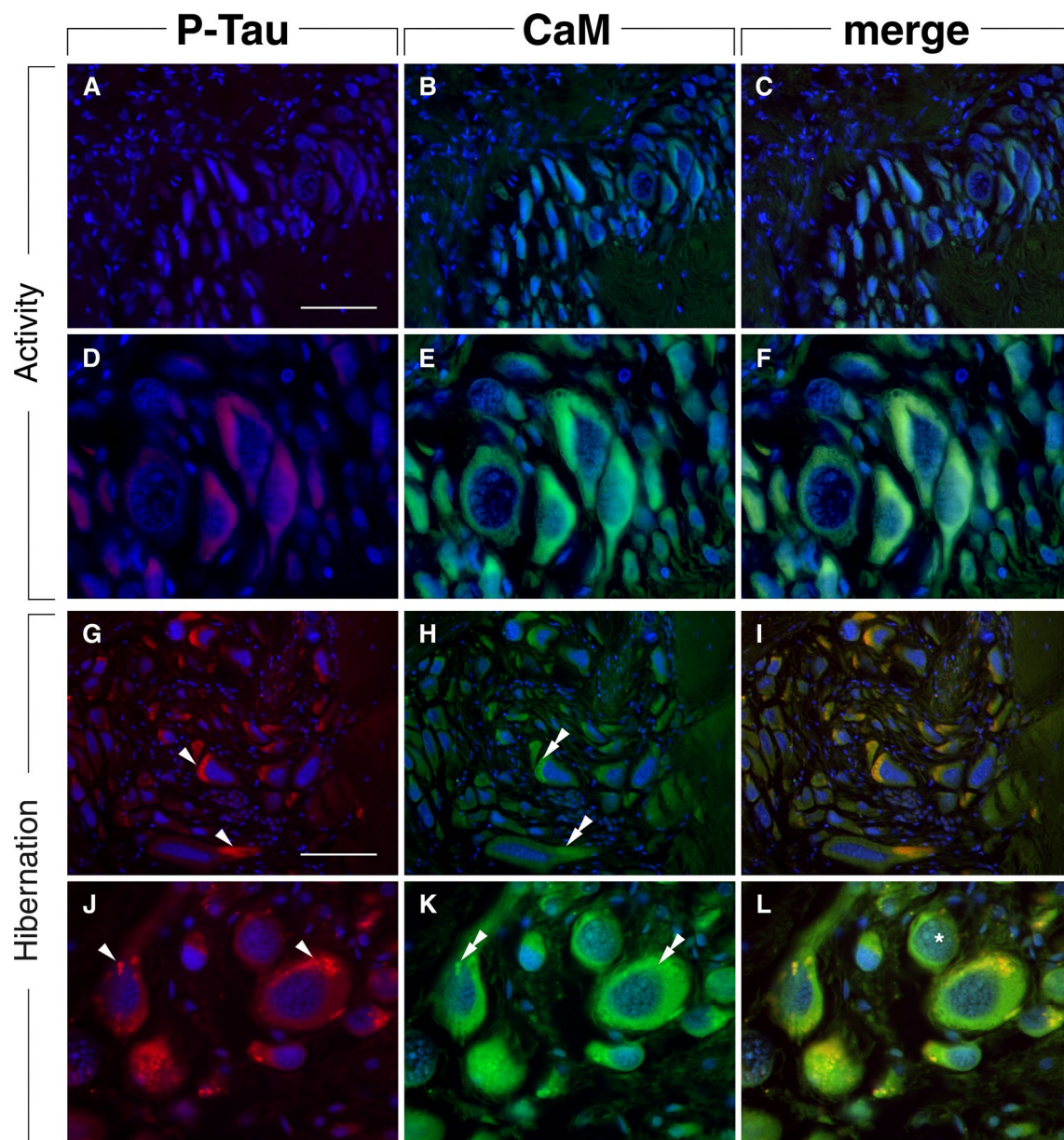


Fig. 4 Micrographs showing double immunofluorescence staining for P-Tau-li and CaM-li in metacerebral lobes from active (a–f) and hibernating (g–l) animals. A pale immunopositivity for both P-Tau-li and CaM-li was detectable during active phase (d, e, respectively); some large neurons showed labelling in their cytoplasm but without any sign of colocalization (c, f). Hibernated snail neurons exhibited

an increased immunoreactivity for both proteins (j, k, respectively); peculiar cytoplasmic aggregates were observed for both P-Tau-li (g, j, arrowheads) and CaM-li (h, k, double arrowheads) and clearly colocalized as shown in the merged panels (i, l); heterochromatin granulations were also observed in some nuclei (l, asterisk). *Objective magnifications*: 60 \times (d–f and j–l); 20 \times (a–c and g–i). Scale bar: 100 μ m

intensity throughout the whole cytoplasm between activity and hibernation. Immunofluorescence intensity significantly increased during the hibernating phase for all the markers considered (Fig. 6a). Active sections incubated with anti-MAP-2 antibodies showed a total mean grey value of 89.532 (SE 1.413). During hibernation the value was significantly higher (mean 116.562; SE 1.631; p value < 0.01). The intensity of CaM-li labelling was low in active animals (mean

74.084; SE 0.900), but significantly increased in hibernating snails (mean 92.911; SE 1.486; p value < 0.01). Particle analysis on NF-H-li immunolabelled active neurons showed a mean grey value of 83.468 (SE 1.391); the fluorescence intensity increased in the hibernated metacerebrum (mean 116.409; SE 1.963; p value < 0.01).

Immunohistochemistry for MAP-like and CaM-li proteins revealed the presence of cytoplasmic aggregates during

Fig. 5 Double immunofluorescence staining for heavy neurofilament-like (NF-H-li) and CaM-li proteins in the metacerebrum during activity (a, b) and hibernation (c–g). Active neurons showed NF-H-li immunopositivity both at the level of the cytoplasm and the nucleus; in the latter, several neurons showed a particularly strong immunoreactivity (a, b, asterisk). During hibernation, a significant increase in cytoplasmic NF-H-li immunoreactivity was detectable in the cytoplasm, while the nuclei completely lacked any sign of positivity (c, d). NF-H-li and CaM-li colocalized in the cytosol of hibernated neurons, including the giant C3 cell, where CaM-li immunolabelled aggregates were detectable (f, g, arrowheads). Objective magnifications: 60× (b, d and e–g); 20× (a, c). Scale bar: 50 μm

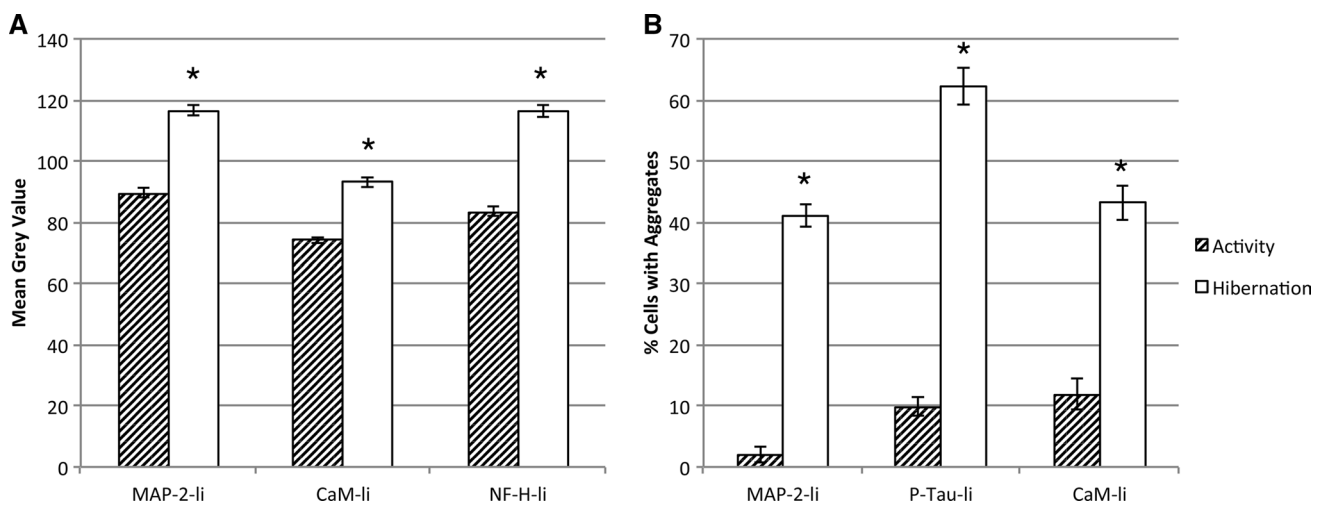
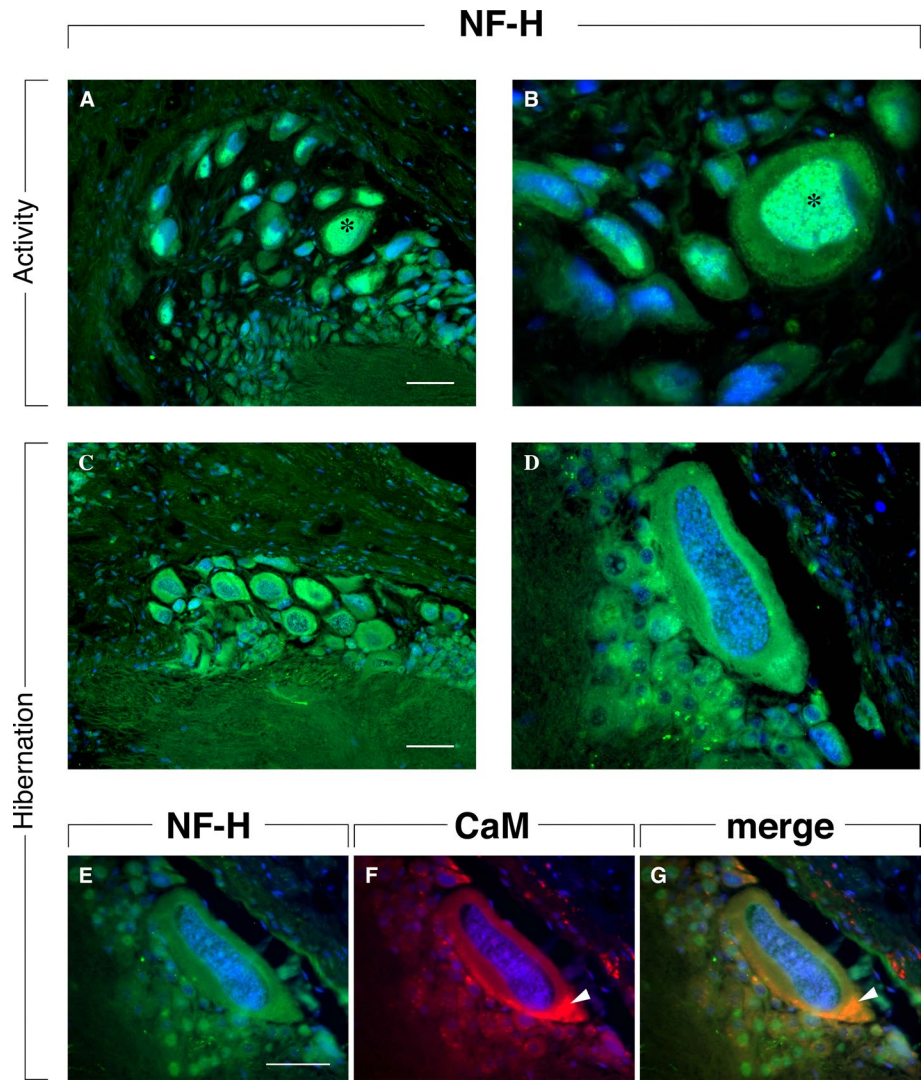


Fig. 6 Comparison of the fluorescence intensity, measured as the mean grey value of each cell (a), and of the percentage of metacerebral neurons showing cytoplasmic aggregates (b) between active

(green) and hibernating (blue) snails. Asterisks indicate highly significant differences between the two phases of the annual cycle (Students' *t* test, *p* value < 0.01)

hibernation; the difference in the number of cells containing aggregates between activity and hibernation was highly significant for all the three proteins considered (Student's *t* test *p* value < 0.01) (Fig. 6b). During activity, less than 2% of all metacerebral neurons showed some level of MAP-2-li aggregates (mean 1.975%; SE 1.22%), and only around 10% contained P-Tau-li (mean 9.84%; SE 1.49%) and CaM-li (mean 11.79%; SE 2.55%) aggregates. Hibernation caused a significant increase in the percentage of cells containing MAP-2-li (mean 41.025%; SE 1.75%), P-Tau-li (mean 62.28%; SE 3.04%) and CaM-li (mean 43.16%; SE 2.78%) cytoplasmic masses.

Discussion

Molluscs are useful models to study the nervous system from a cytological, physiological and pathological point of view, and they have recently been proposed as a valid alternative model in translational medicine (Tascedda et al. 2015). Among the advantages of molluscan models are the large size of their neurons, the simple organization of their neural circuits and the reduced time and costs needed for animal care and experiments. A clear example is the widespread use of gastropods to study neurobiological aspects of memory and synaptic plasticity (Ghirardi et al. 1995; Hawkins et al. 2006; Nikitin and Balaban 2014; Bogodvid et al. 2017). Species of the genus *Cornu* have additionally been used to study cytological modifications accompanying annual cycle changes, particularly those associated with the hibernation phase (Bernocchi et al. 1998; Pisu et al. 1999, 2000). Previous studies have highlighted changes in the immunohistochemical detection of different neural markers, including cytoskeletal proteins, neurotransmitters and neuromodulators (Hiripi and Salánki 1973; Vignola et al. 1995), and in the relationship between neuron and glial cells (Fenoglio et al. 1997). The changes have been linked to decreased neural activity, to accumulation and storage of proteins and precursors in the somata and then to a general plasticity of the CNS (Vignola et al. 1995; Pisu et al. 2000).

In the present study, we focused on immunohistochemical changes in the metacerebrum of hibernated *C. aspersum*. This portion of the cerebral ganglion contains several neuron populations, ranging from medium to giant size, that are associated with very distinct functions, projecting to most of the other ganglia through connective nerves. Activities that are reportedly controlled by metacerebral neurons include feeding, movement and reproduction, all of which are suspended during hibernation, when the electrical activity and possible function of these neurons are still unknown (Chase 2000).

In the analysis and interpretation of data, it is important to consider that a panel of antibodies produced and tested

in mammals was used on cells of a protostome invertebrate species. Despite this, all the proteins considered in this study showed a distinct and neural-specific pattern and have been already described and characterized in different gastropod molluscs (Weber et al. 1991; Kerschbaum et al. 1993; Shemesh et al. 2008; Li et al. 2016. See Results for detailed description), supporting the reliability and specificity of our results and of those already published in the literature.

Because the structural reorganization of neurons is mainly controlled by modifications of the cytoskeleton, we started a deep in situ analysis by characterizing microtubule-associated proteins (MAPs), which have a fundamental role in controlling microtubule assembly and disassembly (Dehmelt and Halpain 2004; Penazzi et al. 2016).

MAP-2 is the most abundant MAP in the vertebrate brain and controls microtubules assembly during neurogenesis and plasticity by stabilizing their polymerized structure (Johnson and Jope 1992). The present results show an increased immunopositivity during hibernation for the employed antibody in the form of aggregates that are concentrated in the cytoplasm of several large neurons, as visible at high magnification in Fig. 2. To understand the possible functional significance of this pattern, we also analysed the expression of another microtubule-associated protein, P-Tau-li. Tau phosphorylation is a mechanism that controls the dynamic balance between assembly and disassembly of microtubules and is therefore of primary importance for neural plasticity (Gómez-Ramos et al. 2004). In humans, pathological hyperphosphorylation of tau leads to microtubule destabilization and formation of neurofibrillary tangles, one of the hallmarks of Alzheimer's disease and other Tauopathies (Iqbal et al. 2009; Bakota and Brandt 2016). Interestingly, in the last two decades non-pathological and reversible tau hyperphosphorylation was also found in conditional and obligate hibernating mammals, such as hamsters, ground squirrels and bears (Arendt et al. 2003; Arendt 2004). These surprising results have been interpreted as neuroprotective mechanisms, which are currently being studied to understand possible relationships and applications with human Tauopathies (Stieler et al. 2011). The neuroprotective hypothesis states that tau phosphorylation is not only essential to allow rearrangements of neural circuits by increasing neural plasticity, but it also contributes to maintain the cell in a controlled and balanced hypometabolic state of "*vita minima*" where cell death is avoided (Arendt and Bullmann 2013). Although the precise molecular mechanisms are still unclear, the evidence that increased tau phosphorylation decreases cell apoptosis strongly supports this hypothesis (Lesort et al. 1997; Esclaire et al. 1998).

Up until now, hyperphosphorylated tau has been investigated only in a few hibernating mammal models. This study provides the first evidence of tau phosphorylation during hibernation in a mollusc. The results show that in cerebral

ganglia of inactive *C. aspersum* tau is highly phosphorylated and concentrated in distinct masses in the cytoplasm of neuronal cell bodies (Fig. 2). These clusters closely resemble the paired helical filaments (PHFs) seen in human Tauopathies, but just like in hibernating mammals they seem to be specific to the dormancy phase and they quickly disappear after arousal, as demonstrated by the identical pattern of immunoreactivity in active and 6-day awakened animals.

Consistently, even though nuclei of hibernating neurons show granular heterochromatin masses that may imply a reduced transcription and consequently reduced cellular activity, no activation of apoptosis was detected in hibernating neurons, as immunoreactions against caspase-3 and caspase-9 showed absence of positivity (data not shown, Ferrari B. personal communication). This seems to indicate that intrinsic mechanisms of apoptosis triggered by neural plasticity or hypometabolism are likely blocked through neuroprotective strategies. Together with the distribution of P-Tau-li, these results suggest that the neuroprotective function of tau might not be exclusive of mammals, but could be a conserved mechanism used by animals to survive during inactivity periods.

The activity of cytoskeletal proteins, including MAPs, is controlled by several molecules that can directly interact with them or activate kinases and phosphatases that subsequently modify them. As the major Ca^{2+} -binding protein in most eukaryotic cells, CaM mediates many basic intracellular processes (Cheung 1978; Chin and Means 2000). It has been shown to directly interact with MAPs with a flip-flop mechanism, by which in the presence of calcium ions CaM forms complexes with MAP-2 and tau and therefore inhibits microtubule assembly (Kakiuchi and Sobue 1981; Lee and Wolff 1984; Padilla et al. 1990). Moreover, tau phosphorylation is controlled by many kinases, including members of the Ca^{2+} /CaM-dependent family (Baudier and Cole 1987; Gupta and Abou-Donia 1998; Su et al. 2008; Wei et al. 2017). Significantly, it has been shown that Ca^{2+} /CaM-dependent kinases overexpression promotes abnormal tau phosphorylation and consequent neurodegeneration in a transgenic *Drosophila* model (Oka et al. 2017).

In the garden snail, CaM-li immunopositivity was known to increase in the CNS during hibernation (Vignola et al. 1995). The results found here confirm those obtained in previous papers for the mesocerebrum (Fig. 3), but through immunofluorescence the presence of calmodulin accumulation in specific areas of the cytoplasm of more than 40% of metacerebral neurons was also detected (Figs. 4 and 5). Interestingly, CaM-li aggregates were also detected in the giant C3 neuron, which has a primary role in tentacle retraction (Prescott et al. 1997). Double immunoreactions with P-Tau-li revealed that the two signals colocalize with similar aggregates, strongly indicating a functional correlation (Fig. 4). Furthermore, the scarcity of calmodulin signal in

active neurons suggests that the condition seen in hibernating ganglia is a regulated phenomenon specific to this stage of the annual cycle. The most likely explanation for the colocalization of these proteins is that calmodulin activates Ca^{2+} /CaM-dependent kinases which in turn phosphorylate tau, leading to PHF-like formation. Supporting this scenario, the activity of at least two Ca^{2+} /CaM-dependent kinases was reported in *Aplysia* neurons (DeRiemer et al. 1984). On the other hand, another scenario can be considered, in which CaM directly interacts with tau and therefore increase neural plasticity by contributing to microtubule disassembly. In both hypotheses, it is clear that metacerebral neurons, including the giant C3 cells, undergo cytochemical changes specific to the hibernation phase in gastropod molluscs. Future studies may help to reveal the functional significance of these changes in the context of the physiological and behavioural modifications associated with hibernation.

In order to understand whether proteins that belong to the same class but have different functions display a different distribution in the two life stages, we analysed the immunoreactivity for *Cornu* PV-li protein, a low molecular weight Ca^{2+} -binding protein characterized by the presence of the EF-hand motif. While CaM is mainly used as an intracellular signalling molecule, PV has a general role in the regulation of intracellular Ca^{2+} homeostasis and electrical activity, avoiding potential intracellular Ca^{2+} concentration impairments that could lead to neuronal degeneration (Hermann et al. 1991; Choi 1992). Our results did not detect any change throughout the annual cycle in the immunopositivity for PV-li, which remains highly expressed in all hibernated metacerebral neurons (Fig. 3), suggesting a conserved involvement of this protein in vital processes related to Ca^{2+} homeostasis that are required for neuron survival regardless of their functional state.

Finally, NFs are another class of cytoskeletal proteins whose production and transport are finely regulated through phosphorylation (Ackerley et al. 2003). They are the main constituents of neuronal cytoskeleton and contribute to their development, shape, stability and transport (Liu et al. 2004). The increased immunolabelling of neurons' cytoplasm using polyclonal antibodies for both the phosphorylated and non-phosphorylated heavy subunit of neurofilaments (NF-H), as shown in Fig. 5, could indicate an additional function in cell stability, as it has been demonstrated that a high concentration of NF-H makes neurofilament proteins more resistant to the action of proteases (Goldstein et al. 1987; Liu et al. 2004). Alternatively, it may indicate NF storage due to a reduced axonal transport.

Previous studies have identified several cytoplasmic intermediate filament (IF) proteins in molluscs and other protostome phyla (Weber et al. 1991). Curiously, the vast majority of these protostome proteins, including most *Cornu* IF proteins, possesses regions that are

shared with vertebrate lamins (Erber et al. 1998). It has been proposed that chordate cytoplasmic IFs, which lack lamin subdomains, arose from an ancestor IF sequence that is still expressed in all protostomes and that the archetypal cytoplasmic IF gene evolved following a loss of the regions specifying for the nuclear localization (Weber 1995; Erber et al. 1998). Therefore, the immunopositivity for NF-H-li in the nuclei of active neurons may be due to cross-reaction with nuclear epitopes corresponding to lamin proteins. If this hypothesis is true, then the absence of positivity in the nuclei of hibernating neurons may reflect a specific autoregulation of IF expression during inactivity. In accordance with this scenario, changes in the structural components of the nucleus were found in the adrenal cortex of hibernating dormice (Malatesta et al. 1995), and the expression of some human lamins appears to be downregulated in cardiomyocytes of chronic hibernating myocardium, a condition in which heart cells are subject to severe hypometabolic conditions (Ausma et al. 1996). CaM-li and NF-H-li colocalized at the level of neurons somata, although the latter did not show any sign of aggregation (Fig. 5). Ca²⁺/CaM-dependent kinases may therefore be one of the kinases involved in NF phosphorylation, but without the typical distribution observed with tau.

In conclusion, the present study shows that hibernation in a mollusc model is characterized by marked functional modifications that are likely associated with neural plasticity and contributes to the species ability to survive potentially harmful environmental conditions. Some of the changes appear to be like those observed in other distantly related hibernating species, such as mammals. This is particularly interesting as it might imply that similar strategies independently evolved at the cellular level to adapt to the same environmental condition. Indeed, if the mechanisms are convergent, pulmonate gastropod molluscs could offer a powerful new tool to investigate cytological similarities with some CNS pathologies affecting humans. In view of this, our present investigation using *C. aspersum* may contribute to underline the protective role of P-Tau during hibernating phases, attempting to link it with cellular mechanisms typical of neurodegenerative disorders such as Tauopathies.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests. The authors have no relevant affiliation or financial involvement with any organization or entity with a financial interest or conflict concerning the information presented in this manuscript.

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